

## MICRO-IODOMETRY OF ALDOSE\*

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In studies on the structure of acidic polysaccharides (4, 5) containing galacturonic acid and several neutral sugars, it was necessary to have a convenient and accurate method for the determination of these sugars.

Many methods have been developed for microanalysis of sugars. However, some of these methods are inadequate for the determination of small amounts of sugars, especially separated by paper chromatography. Colorimetric methods, e.g., the anthrone-sulfuric acid (11) and phenol-sulfuric acid (3) methods, have been widely used for microanalysis of sugars because of their simplicity, accuracy and sensitivity. But these methods also have certain disadvantages. In most color reactions of these methods, different sugars give different degrees of color intensity and, accordingly, the standard curve should be prepared for each of the given sugars. Moreover, the absorbance-concentration relationship is not always linear even at relatively low concentration range, the degree depending on the differences of sugar and method. In contrast, iodometric method is superior in these respects, in which the oxidation with iodine is strictly stoichiometric and different sugars give the same iodine consumption for the equimolar concentration. Preparation of the standard curves, accordingly, is not required for the analysis of different sugars. The usual iodometric procedures (9, 10, 18), however, cannot be applied to the determination of microgram amounts of sugars because of their lower limits of sensitivity. Moreover, this method has been reported to be inapplicable to the determination of certain sugars, such as mannose, rhamnose (7), and higher oligosaccharides (1). Certain works, however, have indicated that mannose can be determined by lengthening the time of reaction (19) or by decreasing the concentration of alkali in the reaction mixture (13).

In 1957 Colbran and Nevell (2) found that the oxidation of glucose was considerably affected by the concentration of potassium iodide of the iodine reagent. Additional information was provided by Miller and Burton (12), who developed a modified iodometric procedure in which the excess of iodine was measured spectrophotometrically and incomplete oxidation of glucose was obtained at low iodide concentrations, whereas at high iodide concentrations the iodine consumption, even with mannose, was almost complete. These results suggest that other difficultly determined sugars would respond similarly, although experimental details regarding the concentration range and effect of iodide are lacking.

In the present paper a modification of the Willstätter-Schudel method (18) is described in which consideration was given to pH, iodine concentration and espe-

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cially potassium iodide concentration. Under appropriate conditions rhamnose, and oligo- and polysaccharides as well as hexose and pentose were oxidized completely by iodine. This method compares favorably in sensitivity and accuracy with the colorimetric method above-mentioned (3).

## EXPERIMENTAL

### 1. Materials

#### *Sugars*

Galactose, arabinose, xylose, rhamnose, lactose (E. Merck A. G.), glucose and maltose (Katayama Chemical Co, Ltd., Japan) were obtained from commercial sources. All the sugars used were chromatographically homogeneous.

#### *Pectic Substances*

All the following pectic substances were prepared from Citrus Pectin purchased from Nippon Kako Co. Ltd.

*Galacturonic acid.* The hydrolysate of pectin by sulfuric acid was neutralized with barium carbonate and filtered. The filtrate was treated with active carbon, made barium-free by passage through a column of cation exchange resin (H-form) and concentrated to a small volume. Galacturonic acid was obtained as a crystalline from the concentrated solution at 4°C. Recrystallization was made from water.

*Di- and trigalacturonic acids.* The digest of pectic acid by the endo-polygalacturonase of *Saccharomyces fragilis* (16) was subjected to the gel filtration on Sephadex G-75 column which resulted in separation of an oligogalacturonide fraction from other products of higher molecular weight and enzymes. Di- and trigalacturonic acids were prepared by treating the oligogalacturonide fraction with DEAE-cellulose as described in a previous paper (4).

*Acid insoluble and acid soluble pectic acids.* These pectic acids were prepared from the partial acid hydrolysate of pectin (15) by DEAE-cellulose column chromatography in the same manner as described previously (6). On the basis of reducing end determination, the average degrees of polymerization of the acid insoluble and acid soluble pectic acids were calculated to be 45.8 and 11.2, respectively.

*4, 5-Unsaturated digalacturonic acid.* Crystalline calcium salt of 4, 5-unsaturated digalacturonic acid was prepared as described previously (14).

*Unsaturated acid soluble pectic acid.* Pectin was degraded in 0.05 N sodium hydroxide at 100°C for 5 minutes (6). The degradation product was fractionated by DEAE-cellulose chromatography in the same manner as described for the acid soluble pectic acid. The average degree of polymerization of the unsaturated pectic acid was found to be 16.2.

#### *Other Chemicals*

All other chemicals, except sodium thiosulfate (c. p., Katayama Chemical Co. Ltd., Japan), were of reagent grade (Ishizu Pharmaceutical Co. Ltd., Japan).

### 2. Procedure for Determination of Aldose

#### *0 to 1 Micromole Range*

To a  $1.6 \times 10$  cm tube 0.5 ml of test solution was transferred, followed by

addition of 1 ml of 0.0025 N iodine in 5% potassium iodide. Next, 0.5 ml of 0.6 M sodium carbonate was added and the tube was immediately closed with a rubber stopper and immersed in water-bath at 20°C for 80 minutes (in the case of rhamnose, held for 200 minutes). At the end of the given time the contents of the tube were acidified with 1 ml of 2 N sulfuric acid and titrated with 0.0005 N sodium thiosulfate containing 0.02% sodium carbonate. As indicator 2 drops of 1% solution of soluble starch were used. A blank test was made with each set of determination, distilled water being used in place of the test solution.

The thiosulfate solution was kept in a brown glass bottle and simple precautions were taken against exposure to bright sunlight.

#### *0 to 4 Micromoles Range*

In a test tube (1.6 × 10 cm), 0.5 ml of test solution was mixed with 1 ml of 0.01 N iodine in 5% potassium iodide and 0.5 ml of 0.6 M sodium carbonate. Next, the tube was allowed to stand for 60 minutes under the same conditions as described above. In case of rhamnose, and oligo- and polysaccharides, the tube was allowed to stand for 180 minutes. Next, the contents of the tube were acidified with 1 ml of 2 N sulfuric acid and titrated with 0.002 N sodium thiosulfate containing 0.02% sodium carbonate, 2 drops of the starch solution being used as indicator. A blank test was made by substituting distilled water for the test solution.

Alternatively 1 ml of test solution was used. In this case the test solution was mixed with 1 ml of 0.01 N iodine in 6% potassium iodide and 0.5 ml of 0.75 M sodium carbonate. Subsequent steps were the same as described above.

## RESULTS

### *1. Conditions for Oxidation of Aldose by Iodine*

#### *pH*

The results given in Fig. 1 show the influence of pH on the oxidation of glucose by 0.0025 N iodine. At pH 11.5, glucose was almost completely oxidized for 50 minutes, while at pH 10.8 and 9.6 with a reaction time of 80 minutes the rate of oxidation was 94 and 27%, respectively. To determine the effects of concentration of alkali, further tests were made with 0.2, 0.4, 0.6, 0.8 and 1 M of sodium carbonate under the conditions shown in Fig. 1. In each case glucose was almost completely oxidized for 50 minutes.

#### *Potassium Iodide Concentration*

Glucose (50  $\mu$ g) was oxidized almost completely by 0.0025 N iodine for 60 minutes, when the concentration of potassium iodide of the iodine reagent was over 4% (Fig. 2). At low iodide concentrations, however, the oxidation of glucose was incomplete; the rates of oxidation for 60 minutes with 2 and 1% iodide were 89 and 49%, respectively. Similar results were obtained when 250  $\mu$ g of glucose was oxidized by 0.01 N iodine (Fig. 3); with 4% iodide the reaction was complete in 30 minutes, but with 1% iodide the reaction proceeded

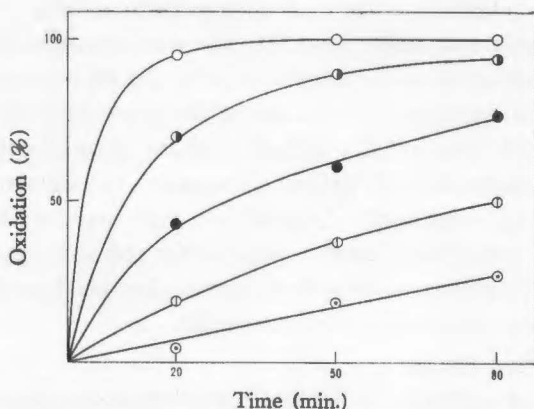


Fig. 1. Effect of pH on oxidation of glucose by iodine. Reaction mixtures containing 0.5 ml of 0.01% glucose, 1 ml of 0.0025N iodine in 5% potassium iodide and 0.5 ml of buffer were kept at 20°C for a given time. Buffers used: —0.6 M Carbonate-bicarbonate buffer, pH 9.6 (○—○), pH 9.9 (□—□), pH 10.2 (●—●), pH 10.8 (◐—◐); 0.6 M sodium carbonate, pH 11.5 (○—○). Details are given in the text.

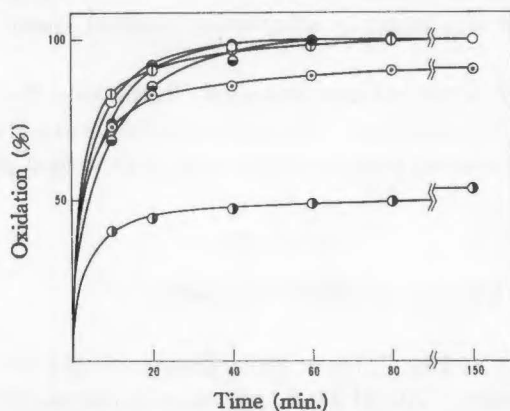


Fig. 2. Effect of potassium iodide concentration on oxidation of glucose by iodine. Reaction mixtures contained 0.5 ml of 0.01% glucose, 1 ml of 0.0025N iodine reagent and 0.5 ml of 0.6 M sodium carbonate. Temperature, 20°C. Potassium iodide concentration of iodine reagent: —1% (●—●), 2% (◐—◐), 4% (□—□), 6% (○—○), 8% (●—●), 10% (●—●).

only to an extent of 75%.

Standard curves were prepared for glucose as shown in Figs. 4 and 5. As can be seen from both figures, the higher the concentration of iodide, the larger amount of glucose was oxidized completely by iodine. With 2, 3, 4 and 5% iodide in 0.0025N iodine reagent the maximum amount of glucose for complete oxidation was about 40, 130, 170 and 180  $\mu$ g (1 micromole), respectively (Fig. 4). In the case of 0.01N iodine reagent the maximum amount of glucose was about 350, 600, 680 and 720  $\mu$ g (4 micromoles), respectively (Fig. 5).

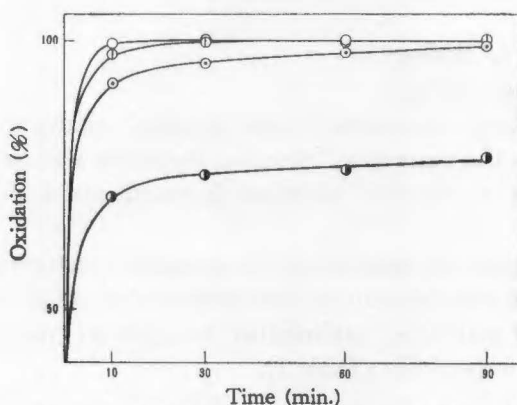


Fig. 3. Effect of potassium iodide concentration on oxidation of glucose by iodine. The conditions are the same as given in Fig. 2 except that the concentrations of glucose and iodine are 0.05% and 0.01N, respectively. Potassium iodide concentration of iodine reagent: —1% (●—●), 2% (◐—◐), 4% (○—○), 6% (○—○).

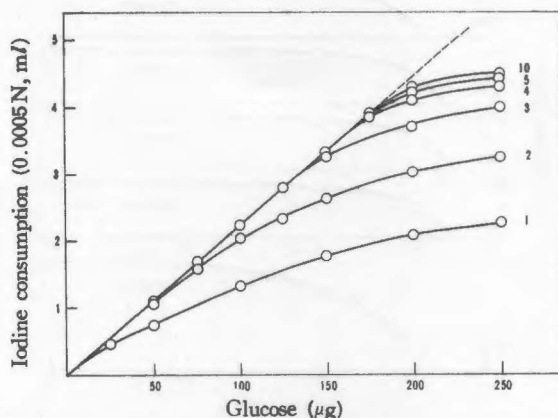


Fig. 4. Standard curves for glucose (0.0025N iodine reagent). Reaction mixtures containing 0.5ml of glucose solution, 1ml of iodine reagent and 0.5ml of 0.6M sodium carbonate were kept at 20°C for 150 minutes. The numbers indicate potassium iodide concentrations (%) of iodine reagent.

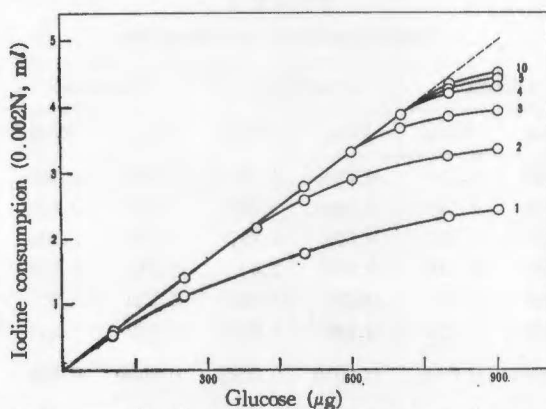


Fig. 5. Standard curves for glucose (0.01N iodine reagent). The conditions are the same as given in Fig. 4 except for the iodine concentration and reaction time (90 minutes).

## 2. Determination of Aldose

### 0 to 1 Micromole Range

All sugars, except rhamnose, were oxidized almost completely for 60 minutes (Fig. 6). In the same time, however, rhamnose was oxidized to an extent of only 83% and for the complete oxidation the reaction time of about 180 minutes was required.

In order to estimate the accuracy of the procedure, determinations of glucose, arabinose, rhamnose and galacturonic acid were carried out at two different sugar concentrations of 10 and 50  $\mu\text{g}$ , and resulted in errors no greater than 1 to 1.5% and 0.2 to 0.5%, respectively (Table 1).

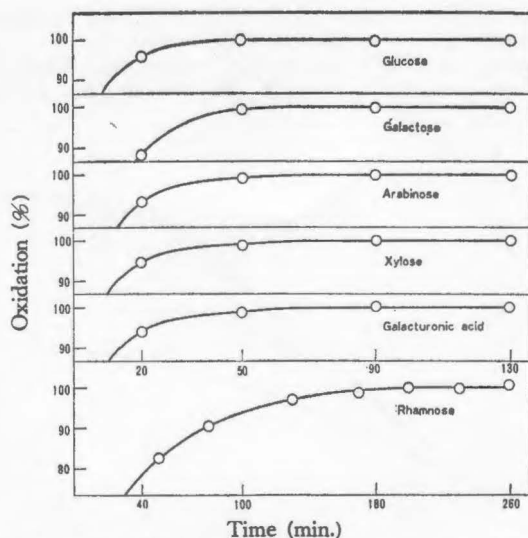


Fig. 6. Rate and degree of oxidation of aldoses with iodine. Aldoses (100  $\mu\text{g}$ ) were oxidized by 0.0025N iodine reagent containing 5% potassium iodide. Details are given in the text.

TABLE 1  
Reproducibility of titrations

| Determination      | Glucose          |                  | Arabinose        |                  | Rhamnose         |                  | Galacturonic acid |                  |
|--------------------|------------------|------------------|------------------|------------------|------------------|------------------|-------------------|------------------|
|                    | 10 $\mu\text{g}$ | 50 $\mu\text{g}$ | 10 $\mu\text{g}$ | 50 $\mu\text{g}$ | 10 $\mu\text{g}$ | 50 $\mu\text{g}$ | 10 $\mu\text{g}$  | 50 $\mu\text{g}$ |
| 1                  | 0.228            | 1.118            | 0.265            | 1.337            | 0.246            | 1.215            | 0.215             | 0.973            |
| 2                  | 0.226            | 1.124            | 0.269            | 1.332            | 0.248            | 1.230            | 0.213             | 0.976            |
| 3                  | 0.222            | 1.127            | 0.263            | 1.337            | 0.238            | 1.232            | 0.212             | 0.976            |
| 4                  | 0.227            | 1.116            | 0.272            | 1.340            | 0.243            | 1.218            | 0.219             | 0.974            |
| 5                  | 0.227            | 1.126            | 0.273            | 1.342            | 0.250            | 1.225            | 0.220             | 0.970            |
| Average            | 0.225            | 1.122            | 0.268            | 1.338            | 0.245            | 1.224            | 0.216             | 0.974            |
| Standard deviation | 0.0022           | 0.0042           | 0.0036           | 0.0028           | 0.0036           | 0.006            | 0.003             | 0.0018           |
| Error (%)          | 0.98             | 0.37             | 1.34             | 0.21             | 1.47             | 0.49             | 1.39              | 0.18             |

Values represent ml of 0.0005 N iodine required. Details are given in the text.

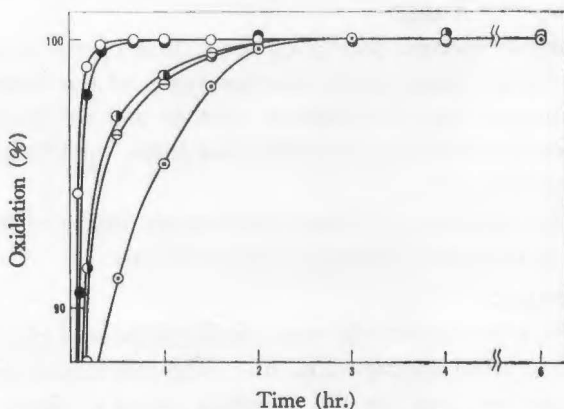


Fig. 7. Rate and degree of oxidation of aldoses with iodine. Aldoses ( $250\mu\text{g}$  for monosaccharides and  $500\mu\text{g}$  for disaccharides) were oxidized by  $0.01\text{N}$  iodine, the volume of test solutions used being  $0.5\text{ml}$  in all cases except for glucose.  $\circ-\circ$  glucose,  $0.5\text{M}$ ;  $\bullet-\bullet$  glucose,  $1\text{M}$ ;  $\odot-\odot$  rhamnose,  $\bullet-\bullet$  maltose,  $\ominus-\ominus$  lactose. Details are given in the text.

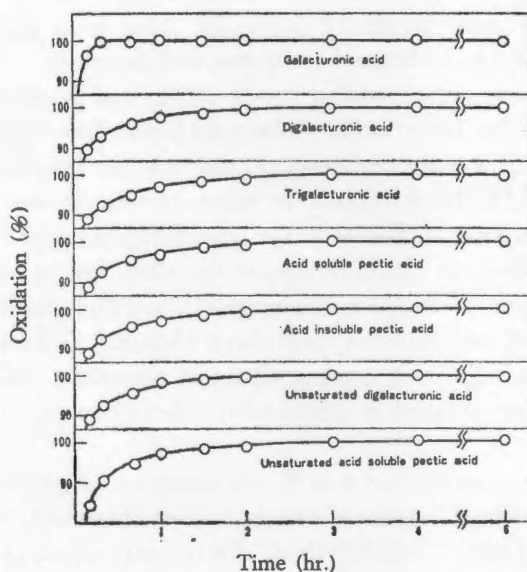


Fig. 8. Rate and degree of oxidation of pectic substances with iodine. Reaction mixtures contained  $1\text{ml}$  of test solution,  $1\text{ml}$  of  $0.01\text{N}$  iodine in  $6\%$  potassium iodide and  $0.5\text{ml}$  of  $0.75\text{M}$  sodium carbonate. In all cases, except for galacturonic acid, the degree of oxidation at the end of 180 minutes was taken to correspond to  $100\%$  reaction. Amounts of the samples used: — Galacturonic acid,  $250\mu\text{g}$ ; digalacturonic acid, about  $0.5\text{mg}$ ; trigalacturonic acid, about  $0.75\text{mg}$ ; acid soluble pectic acid, about  $2.5\text{mg}$ ; acid insoluble pectic acid, about  $10\text{mg}$ ; unsaturated digalacturonic acid, about  $0.5\text{mg}$ ; unsaturated acid soluble pectic acid, about  $2.5\text{mg}$ .



### *0 to 4 Micromoles Range*

In the oxidation of glucose, increasing the volume of test solution from 0.5 to 1 ml had no significant effects on the reaction rate and the oxidation was almost complete in 40 minutes (Fig. 7). Maltose, lactose and rhamnose, as compared with glucose, were oxidized at considerably low rates; in 150 minutes their reactions were complete (Fig. 7).

A typical determination of glucose had average errors of 0.36% (samples, 100  $\mu$ g) and 0.17% (samples, 250  $\mu$ g) on five replicates.

### *Pectic Substances*

The results for galacturonic acid were similar to those of glucose; the reaction was almost complete in 40 minutes (Fig. 8). With other uronides, however, the reaction rates were low and for the complete reaction times of 120 to 150 minutes were required.

## DISCUSSION

### *Conditions for Oxidation of Aldose by Iodine*

Oxidation of glucose proceeded at a maximal rate when sodium carbonate was used as alkali agent at pH 11.5 (Fig. 1). This is in good agreement with the optimum pH of 11.3 obtained by Ingles and Israel (8). The use of sodium carbonate as alkali was recommended also by Miller and Burton (12) and Macleod and Robison (10). The latter authors observed incomplete oxidation of glucose at the concentrations of sodium carbonate in the reaction mixture above the range from 0.016 to 0.03 M. To determine the effects of concentration of alkali, glucose was oxidized with sodium carbonate in the concentration range from 0.05 to 0.25 M. However, the different concentrations of the alkali had no significant influence on the rate and degree of oxidation of glucose. The difference between the results obtained by Macleod and Robison, and those obtained in the present study may be due to the use of different concentrations of potassium iodide in the reaction mixture, because the oxidation is considerably affected by the iodide concentration (2).

As can be seen from Figs. 2 and 3, the oxidation of glucose is incomplete at low iodide concentrations. Similar results have been obtained by Miller and Burton (12). According to Colbran and Nevell (2) the incompleteness of glucose oxidation is attributable to the rapid decomposition of iodine in alkaline solutions which takes place especially at low iodide concentrations; with 0.8% potassium iodide at pH 10.8 and 20°C, nearly all the active agent (i. e., free iodine) in 0.01 N iodine solution decomposed into iodate and iodide within 5 hours, whereas with 8% iodide a considerable amount of iodine remains in active state even after two days. At low iodide concentrations, however, the complete oxidation of glucose can be obtained by using several times the theoretical amount of iodine. For example, with 1% iodide in the reaction mixture glucose was oxidized completely by 0.0025 and 0.01 N iodine reagent using about 5.6 and 2.6 times the theoretical amount



of iodine, respectively (Figs. 4 and 5). From both figures it can also be seen that the higher the concentration of the iodine reagent, the less effect of potassium iodide on the degree of oxidation is observed. However, regardless of the concentration of iodine reagent, glucose can be oxidized completely by 1.25 times the theoretical amount of iodine, provided that the concentration of iodide in the reaction mixture is over 2.5 %.

The useful range of the iodine reagent can be extended by increasing the iodide concentration. On the other hand, at high iodide concentrations secondary reactions (over-oxidation) with sugars may take place (2, 12). At 2.5 % iodide in the reaction mixture, however, no secondary reaction occurred with different sugars at least within 6 hours (Figs. 7 and 8).

#### *Determination of Aldose*

The time required for complete reaction of aldose with iodine depends upon the structure of aldose. As can be seen from Figs. 6 to 8, hexose, pentose and galacturonic acid were oxidized completely 2.5 to 3 times faster than rhamnose, and oligo- and polysaccharides. On the basis of these data, the reaction time for the determination of the first group aldoses, such as hexose, pentose and galacturonic acid, was set at 80 and 60 minutes for 0.0025 and 0.01N iodine reagent, respectively. For the second group aldoses, such as rhamnose, and oligo- and polysaccharides, the reaction time of 200 and 180 minutes was chosen for iodine concentrations of 0.0025 and 0.01N, respectively.

With 0.0025N iodine reagent, determinations of glucose, arabinose, rhamnose and galacturonic acid were carried out at two different concentrations of 10 and 50  $\mu\text{g}$  of each sugar, and resulted in errors no greater than 1 to 1.5 % and 0.2 to 0.5 %, respectively. The errors obtained by Dubois *et al.* by the phenol-sulfuric acid method for galactose of 20, 40 and 80  $\mu\text{g}$  are 0.5, 1.7 and 1.1 %, respectively (3). This iodometric procedure, therefore, compares favorably in sensitivity and accuracy with the colorimetric method. This procedure is particularly well adapted to the determination of aldoses separated by paper chromatography.

The reagent of 0.01N iodine has a relatively wide useful range and can be used for the determination of as much as 720  $\mu\text{g}$  of glucose and other aldoses of equivalent reducing power. This procedure may be expected to be useful for the determination of reducing sugars in biochemical investigations.

Since the works of Hirst *et al.* (7) and Bailey *et al.* (1) iodometric method has often been assumed to have a serious limitation (17) because of the uncertainty of the stoichiometry of iodine consumption with certain sugars, such as mannose, rhamnose (7), and higher oligosaccharides (1). However, the sugars which have been considered difficult to determine are oxidized completely by iodine under the conditions described in this paper. The difference between the results reported by the above authors and those obtained in the present study may be due to the use of different concentrations of potassium iodide in the reaction mixture, because, as pointed out by Colbran and Nevell (2), the oxidation reaction is considerably affected by the concentration of potassium iodide.

The iodometric method has been, and is still being, widely used, but little attention is paid to the iodide concentration. The present results indicate that aldose can be oxidized completely by 1.25 times the theoretical amount of iodine, provided that the concentration of iodide in the reaction mixture is over 2.5%.

#### SUMMARY

The conditions for the determination of very small amounts of aldoses by the iodometric method were investigated.

1) The oxidation of glucose by iodine was influenced to a marked extent by the concentration of potassium iodide in the reaction mixture. At too low iodide concentration it was incomplete, but complete at 2.5% concentration or higher.

2) Glucose could be oxidized completely by 1.25 times the theoretical amount of iodine, provided that the concentration of iodide in the reaction mixture was over 2.5%.

3) The time required for complete reaction of aldose with iodine depended upon the structure of aldose. Hexose, pentose and galacturonic acid were oxidized completely 2.5 to 3 times faster than rhamnose, and oligo- and polysaccharides.

4) Determinations of glucose, arabinose, rhamnose and galacturonic acid resulted in errors no greater than 1 to 1.5% (samples, 10  $\mu$ g) and 0.2 to 0.5% (samples, 50  $\mu$ g).

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